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**APPLICATION NUMBER:** *60/555,177*
**FILING DATE:** *March 22, 2004*
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18351 U.S. PTO 032204

# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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15535 U.S.PTO 60/555,177 032204

| INVENTOR(S) | | |
|---|---|---|
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| Michael A. | Caligiuri | Columbus, OH |

*Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

**TITLE OF THE INVENTION (500 characters max)**

Methods For Transducing Natural Killer Cells

*Direct all correspondence to:* **CORRESPONDENCE ADDRESS**

☑ Customer Number: 24024

*OR*

| | | | | | |
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**ENCLOSED APPLICATION PARTS *(check all that apply)***

☑ Specification *Number of Pages* 23
☐ CD(s), Number
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☐ Application Date Sheet. See 37 CFR 1.76

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☑ Applicant claims small entity status. See 37 CFR 1.27.
☑ A check or money order is enclosed to cover the filing fees.
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☐ No.
☑ Yes, the name of the U.S. Government agency and the Government contract number are: NIH, #P01 CA95426 and #R01 CA68458



*Respectfully submitted,*

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Date March 22, 2004

REGISTRATION NO. 42,920 *(if appropriate)*

Docket Number: 22727/04229

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Docket Number 22727/04229

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Number 1 of 1

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**TOTAL AMOUNT OF PAYMENT** ($) 80.00

| *Complete if Known* | |
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| Application Number | To Be Determined |
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| First Named Inventor | Caigiuri, Michael A. |
| Examiner Name | To Be Determined |
| Art Unit | To Be Determined |
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| 1002 | 340 | 2002 | 170 | Design filing fee | |
| 1003 | 530 | 2003 | 265 | Plant filing fee | |
| 1004 | 770 | 2004 | 385 | Reissue filing fee | |
| 1005 | 160 | 2005 | 80 | Provisional filing fee | 80.00 |

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| | | Extra Claims | | Fee from below | Fee Paid |
|---|---|---|---|---|---|
| Total Claims | | -20** = | | X | = |
| Independent Claims | | - 3** = | | X | = |
| Multiple Dependent | | | | | = |

| Large Entity Fee Code | Large Entity Fee ($) | Small Entity Fee Code | Small Entity Fee ($) | Fee Description |
|---|---|---|---|---|
| 1202 | 18 | 2202 | 9 | Claims in excess of 20 |
| 1201 | 86 | 2201 | 43 | Independent claims in excess of 3 |
| 1203 | 290 | 2203 | 145 | Multiple dependent claim, if not paid |
| 1204 | 86 | 2204 | 43 | ** Reissue independent claims over original patent |
| 1205 | 18 | 2205 | 9 | ** Reissue claims in excess of 20 and over original patent |

SUBTOTAL (2) ($)

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|---|---|---|---|---|---|
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| 1052 | 50 | 2052 | 25 | Surcharge - late provisional filing fee or cover sheet | |
| 1053 | 130 | 1053 | 130 | Non-English specification | |
| 1812 | 2,520 | 1812 | 2,520 | For filing a request for *ex parte* reexamination | |
| 1804 | 920* | 1804 | 920* | Requesting publication of SIR prior to Examiner action | |
| 1805 | 1,840* | 1805 | 1,840* | Requesting publication of SIR after Examiner action | |
| 1251 | 110 | 2251 | 55 | Extension for reply within first month | |
| 1252 | 420 | 2252 | 210 | Extension for reply within second month | |
| 1253 | 950 | 2253 | 475 | Extension for reply within third month | |
| 1254 | 1,480 | 2254 | 740 | Extension for reply within fourth month | |
| 1255 | 2,010 | 2255 | 1,005 | Extension for reply within fifth month | |
| 1401 | 330 | 2401 | 165 | Notice of Appeal | |
| 1402 | 330 | 2402 | 165 | Filing a brief in support of an appeal | |
| 1403 | 290 | 2403 | 145 | Request for oral hearing | |
| 1451 | 1,510 | 1451 | 1,510 | Petition to institute a public use proceeding | |
| 1452 | 110 | 2452 | 55 | Petition to revive - unavoidable | |
| 1453 | 1,330 | 2453 | 665 | Petition to revive - unintentional | |
| 1501 | 1,330 | 2501 | 665 | Utility issue fee (or reissue) | |
| 1502 | 480 | 2502 | 240 | Design issue fee | |
| 1503 | 640 | 2503 | 320 | Plant issue fee | |
| 1460 | 130 | 1460 | 130 | Petitions to the Commissioner | |
| 1807 | 50 | 1807 | 50 | Processing fee under 37 CFR 1.17(q) | |
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| SUBMITTED BY | | | | (Complete *(if applicable)*) | |
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| *Signature* | Sean Myers-Payne | | | *Date* | March 22, 2004 |

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Attorney Docket No.: 22727/04229

UNITED STATES PROVISIONAL PATENT APPLICATION

FOR

# METHODS FOR TRANSDUCING NATURAL KILLER CELLS

BY

MICHAEL A. CALIGIURI

ROSSANA TROTTA

JIANHUA YU

BRIAN BECKNELL

## DESCRIPTION OF THE INVENTION

[001] Research leading to the present invention was funded, at least in part, by NIH Grant Nos. P01CA95426 and R01CA68458. The government has certain rights in this invention.

### Field of the Invention

[002] The invention generally relates to methods for transducing eukaryotic cells, such as natural killer (NK) cells. The methods involve the use of a retroviral expression vector, such as PINCO, and a packaging cell line, such as Phoenix cells.

### Background of the Invention

[003] The innate immune system represents the human body's essential first line of defense against cancer as well as infectious disease. In the immune competent host, innate immune effectors act rapidly to restrict the dissemination of disease, as well as to trigger the adaptive, or antigen-specific, immune system. Natural killer (NK) cells are $CD56^{+}CD3^{-}$ large granular lymphocytes that constitute one component of the innate immune system. In addition to their potent cytolytic activity, NK cells elaborate a host of immunoregulatory cytokines and chemokines, which play a crucial role in pathogen clearance. In particular, NK cells produce nanogram quantities of gamma interferon (IFN-$\gamma$), a critical cytokine for the clearance of infectious pathogens as well as for tumor surveillance.

[004] In rodent models, NK cells have been proven effective for the clearance of certain tumors, as well as bacterial, fungal, viral, and parasitic infections. Furthermore, in rare cases of human congenital immune deficiencies, the absence of NK cells produces a clinical spectrum that parallels classical severe

combined immunodeficiency (SCID) syndromes. The importance of NK cells is magnified in a host of clinical scenarios in which the adaptive immune system is compromised. These states include congenital immune disorders, iatrogenic immune suppression following organ transplantation, and the Acquired Immune Deficiency Syndrome (AIDS). Natural killer cells represent an attractive target for therapeutic manipulation to fight the rampant opportunistic infections and virus-induced cancers that arise under these states of adaptive immunoparalysis. Indeed, this is the rationale underlying ultra low-dose interleukin-2 therapy to heighten cytokine production and potentiate the anti-tumor effects of NK cells in AIDS-associated malignancies (Fehniger *et al.*, 2002). This approach is further substantiated by recent advances in bone marrow transplantation, in which donor-derived NK have been shown to mediate a potent graft versus tumor effect in acute myeloid leukemia (Ruggeri *et al.*, 2002). In view of these advances, a greater mechanistic understanding of NK cells and the innate immune system is needed for providing new means to enhance the function of these cells for the benefit of the immunocompromised patient.

[005] In order to achieve this level of understanding, it is essential to genetically manipulate NK cells. It is only through such experimentation can one discern the role of specific gene products in the signal transduction pathways that govern NK cell behavior. Up to this point, however, the transduction of genetic material into NK cells has presented a major technical hurdle. While some success has been enjoyed with vaccinia vectors, this technique is limited to short-term experiments, given the lytic nature of poxvirus infections. Moreover, since

poxviruses exert a general negative influence on nuclear function, cellular transduction with these vectors severely hampers studies of transcription. More recently, the refinement of electroporation methodologies for NK cells has offered an alternative to viral vectors (Trompeter *et al.*, 2003). However, our own experience with this technology is that it is severely limited by DNA size and choice of DNA vector (B. Becknell, unpublished observations).

[006] Thus, there exists a need in the art for methods for stably transducing NK cells. The present invention answers that need.

## SUMMARY OF THE INVENTION

[007] Features and Advantages of the Invention

[008] This invention presents a novel retrovirus-based method for transduction of human natural killer (NK) cells with genetic material. Unlike previous approaches, this technique results in successful transduction of the $CD56^{dim}$ NK population that predominates among human peripheral blood NK and which is the cellular effector of antibody-dependent cellular cytotoxicity (ADCC). The present invention also results in stable transduction of the $CD56^{bright}$ NK subset as well as NK-derived cell lines.

[009] The present inventive facile approach to NK infection requires less time and cytokines than previously reported. The inventive methodology can be applied to study specific genetic pathways in NK cells and also finds use in the genetic modification of NK cell populations for enhanced therapeutic efficacy in patients with malignancies that are highly susceptible to such immunotherapeutic intervention.

[010] Up to this point in time, technical hurdles have prevented efficient retroviral transduction of primary NK cells and NK-derived cell lines. The advantages of this invention over other methodologies for genetic manipulation of human NK cells are summarized by at least the following three points:

[011] (1) This retrovirus-based approach results in the permanent transduction of NK cells with genetic material. This is in contrast to episomal vectors that are lost with cell division / long-term culture and poxvirus vectors that inhibit nuclear function and eventually instigate host cell lysis.

[012] (2) Unlike other retroviral infections of NK cells described in the scientific literature, our approach is the first to result in successful transduction of the $CD56^{dim}$ NK population.

[013] (3) We demonstrate transduction of a variety of genes into primary NK and NK-derived cell lines, with expression of a marker for infection (the green fluorescent protein) as well as the proteins undergoing study in our laboratory.

[014] Finally, with the advent of NK-cell transplantation in cancer therapy for patients with acute myeloid leukemia, the genetic manipulation of NK cell populations prior to administration will provide therapeutic benefit for the patient - by enhancing NK cell survival, cytolytic function, cytokine production, and/or tumor specific killing.

[015] Additional features and advantages of the invention will be set forth in part in the description that follows, and in part will be obvious from the description, or may be learned by practice of the invention. The features and advantages of the

invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[016] Summary of the Invention

[017] The present invention is directed to a facile, reproducible methodology for retroviral transduction of human primary NK cells and NK-derived cell lines. Large cDNAs (up to about 3.8 kilobases (kB) in length) and the green fluorescent protein (GFP, used as a marker of infection) have been simultaneously expressed using the inventive methods. In contrast with other studies reporting retroviral infection of primary NK, the present inventive approach permits the transduction of the $CD56^{dim}$ NK subset that predominates in human peripheral blood.

[018] The invention provides methods for stably transducing mammalian natural killer cells by: transfecting a packaging cell line with a retroviral expression vector; culturing the transfected packaging cell line in a cell culture medium; and culturing the mammalian natural killer cells with the cell culture medium. In some embodiments, the packaging cell line is chosen from a Phoenix cell line, which can be Phoenix-Ampho. In some embodiments, the retroviral expression vector is PINCO. In some embodiments, the invention further includes separating the transfected packaging cell line from the cell culture medium in which the cell line is cultured prior to culturing the mammalian natural killer cells with the cell culture medium.

[019] The invention also relates to non-naturally occurring mammalian natural killer cells, stably transduced, which express at least one of green fluorescent protein and CD8. The invention is also directed to progeny cell lines of

these non-naturally occurring mammalian natural killer cells, wherein the cell line is polyclonal.

[020] The invention also relates to non-naturally occurring mammalian natural killer cell lines, which stably express their genomes through at least two, four, or eight population doublings.

[021] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

[022] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and together with the description, serve to explain the principles of the invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

[023] **Figure 1** demonstrates infection of primary natural killer cells with PINCO, PINCO-HLX (1.5kb cDNA) and PINCO-SHIP1 (3.8kb cDNA), evaluated by flow cytometry. Numbers in the crosshairs indicate percentage of events for each quadrant.

[024] **Figure 2** demonstrates infection of primary IL-2/KL expanded CD56bright natural killer cells with PINCO and PINCO-dnTBET (2.0 kb cDNA), evaluated by flow cytometry. Numbers in the crosshairs indicate percentage of events for each quadrant.

[025] **Figure 3** demonstrates infection of the NK-92 cell line with PINCO, PINCO-dnTBET (2.0 kb cDNA), PINCO-LDB1 (1.1 kB cDNA), PINCO-TSC22 (0.5 kB cDNA), and PINCO-SHIP1 (3.8 kB cDNA), evaluated by flow cytometry.

[026] **Figure 4** demonstrates infection of the NKL cell line with PINCO, PINCO-TSC22 (0.5 kb cDNA), and PINCO-SHIP1 (3.8 kB cDNA), evaluated by flow cytometry.

[027] **Figure 5** demonstrates transduction of T (CD3+) and B lymphocytes (CD19+) with PINCO Or PINCO-TSC22.

[028] **Figure 6** shows Western blot analysis of FACS purified, virally transduced NK cell lines confirms overexpression of proteins of interest (SHIP, On the left, and LDB1, on the right).

[029] **Figure 7** shows an analysis of interferon gamma (IFN-g) production by PINCO Infected NK-92 cells by intracellular staining (A, in response to IL-12 and IL-18 stimulation) or ELISA (B, in response to indicated stimuli).

[030] **Figure 8** illustrates that PINCO-infected NK-92 cells display cytolytic activity.

## DESCRIPTION OF THE EMBODIMENTS

[031] Reference will now be made in detail to specific embodiments (exemplary embodiments) of the invention, examples of which are illustrated in the accompanying drawings.

[032] The present invention is directed to methods of stably introducing foreign nucleic acids into mammalian cells, including for example, lymphocytic cells. The mammalian cells that can be transformed include, but are not limited to, natural killer cells. Natural killer cells include, but are not limited to, primary NK cells, NK-92, NKL, NK-cell subpopulations, including $CD56^{bright}$ and $CD56^{dim}$ NK cells, and NK-

derived cells, which further includes lymphokine-activated killer (LAK) cells, which are cytokine-activated and cytokine-expanded NK cells, and NK clones.

[033] The foreign nucleic acids are introduced into the mammalian cells by a process involving a retroviral expression vector and a packaging cell line. Basically, the retroviral expression vector is transfected into the packaging cell line, which then allows the vector to propagate. When the mammalian cell line is placed in the presence of the propagated retrovirus, infection occurs, thereby introducing the foreign nucleic acid into the mammalian cell.

[034] The retroviral expression vectors that can be used in accordance with the present invention include, for example, those based on the Epstein-Barr virus (EBV), which can produce episomal propagation of the plasmid. Examples of this type of retroviral expression vectors include, but are not limited to, PINCO.

[035] Foreign nucleic acid sequences are introduced into the expression vectors using conventional techniques.

[036] Packaging cell lines that can be used in accordance with the present invention include, but are not limited to, Phoenix packaging cell lines, which further include, but are not limited to, ecotropic (Phoenix-ECO) and amphotropic (Phoenix-Ampho). Other examples include the commercially available BD Biosciences lines, including BD RetroPack, BD AmphoPack, and BD EcoPack, as well as PA317, an NIH 3T3-based, amphotropic, packaging cell line, Apex, derived from ECV304 (human umbilical vein endothelial cell line HUVECS) / T24 (human bladder cell line), BOSC23, which is derived from HEK 293 T-cells, and PG13, which is derived from TK-NIH/3T3 (mouse fibroblast) cells.

[037] The packaging cell lines are infected with the retroviral vector using conventional techniques.

[038] The transfected packaging cell line is cultured until a sufficiently high viral titer is generated. Determination of the sufficiency of the viral titers is well within the level of one of ordinary skill, and the determination can be made empirically. In some embodiments, the transfected packaging cells are cultured for greater than or equal to about 8 hours, or 16 hours, or one day, or two days, or three days or more. The culturing can be maintained until the viral titer reaches $10^4$, $10^5$, $10^6$, $10^7$, or higher.

[039] When a sufficiently high titer is achieved, the mammalian cells can be transduced using the cell culture medium containing the retroviral vector. This process is performed using conventional culture techniques.

[040] Mammalian cell lines transduced according to the present invention are stably transfected. That is, the foreign nucleic acid introduced into the genome is passed on to progeny cells. Thus, the foreign nucleic acid is present in the first doubled population of cells, along with the third, fourth, fifth, etc., to as many as 20 or more doublings. As doubling time may be several days to a week, the foreign nucleic acid will be present in progeny cell lines for more than a week, or two weeks, or three weeks, or one month, or two months, or six months, or one year, or more.

[041] Examples

[042] I. Generation of retrovirus and lentivirus.

[043] A. Generation of PINCO Retrovirus.

[044] The PINCO retroviral transfer plasmid, originally from the laboratory of Dr. P.G. Pelicci (Grignani *et al.*, 1998), was obtained through the courtesy of Dr. Martin Sattler (Dana Farber Cancer Institute, Boston, Mass). This retroviral vector permits the expression of a gene of interest from the 5′ long term repeat (LTR) as well as GFP from an internal cytomegalovirus (CMV) immediate early promoter. Complementary DNA (cDNA) from genes of interest ranged in size from 0.5 to 3.8 kB and was cloned into the BamHI and/or EcoRI sites of PINCO. Following confirmation of cloning by DNA sequencing, each construct was prepared for virus production by endotoxin-free maxiprep (Qiagen, Carlsbad, California). A plasmid expressing the VSV-G protein (pVSV-G) was similarly prepared.

[045] VSV-G pseudotyped retroviral particles were generated by transient transfection of the Phoenix-Ampho packaging line. Early passage Phoenix cells were cultured (37°C / 5% $CO_2$) on T75 flasks in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with GlutaMAX, antibiotic/antimycotic and 10% fetal bovine serum (all from Invitrogen), hereafter denoted D-10. Phoenix cells were transfected at approximately 80% confluence, after having replaced the medium with D-10 containing chloroquine (Sigma) at 25 μM final concentration. Twenty micrograms (μg) of PINCO and 0.9 μg of pVSV-G were cotransfected into Phoenix cells using the PROFECTION® Mammalian Transfection System—Calcium Phosphate (Promega). Transfected cells were cultured for 12-16 hours at 37°C / 5% $CO_2$.

[046] Thereafter, the medium was replaced with RPMI supplemented with GlutaMAX, antibiotic/antimycotic, and 20% fetal bovine serum, hereafter denoted

RPMI-20. Cells were returned to 37°C / 5% $CO_2$ for another 24 hours. Then, virus-containing supernatant was aseptically filtered through 0.45-μm cellulose acetate (Corning), aliquoted and frozen at -80°C.

[047] II. Transduction of primary human NK cells, NK-derived cell lines, and other lymphocyte populations.

[048] A. Transduction of Primary NK Cells.

[049] Human peripheral blood leukocytes were obtained as discarded buffy coats from the American Red Cross. NK cells were enriched by rossetting (which is a negative selection strategy using bivalent antibodies simultaneously targeting cells of an unwanted lineage (i.e., T, B, and monocytes) and red blood cells, permitting depletion of these cells upon Ficoll centrifugation; Stem Cell Technologies) and Ficoll-Hypaque density centrifugation. Following removal of monocytes by plastic adherence, the preparation contains 2.5 x $10^7$ to 1x$10^8$ peripheral blood mononuclear cells (PBMC), of which approximately 80% are NK as revealed by flow cytometric analysis of the surface antigens CD56 and CD3 (Human NK cells are $CD56^{bright/dim}CD3^{neg}$). This enriched NK (eNK) preparation was cultured for 48 hours at 37°C / 5% $CO_2$ in RPMI-20 supplemented with recombinant human interleukin-2 (IL-2, Roche) at a final concentration of 900 international units (IU) / ml.

[050] Next, between 1-5x$10^6$ eNK were harvested by centrifugation, resuspended in 2 ml viral supernatant supplemented with IL-2 (900 IU/ml) and polybrene (Sigma, 8 ug/ml), and placed in one well of a 6-well tissue-cultured treated plate (Fisher). The plate was centrifuged in a microcarrier bucket at 1800 rpm for 45 minutes at 32°C (Beckman). Following a 2 hour incubation at 32°C / 5% $CO_2$,

medium was gently removed by pipetting using a P1000 and replaced with an additional 2 ml of viral supernatant, supplemented as described. The plate was re-centrifuged and returned to 32°C / 5% $CO_2$ for another 4 hours. Next, the medium was gently removed and replaced with RPMI-20 containing IL-2 (900 IU/ml), and cells were incubated overnight at 37°C / 5% $CO_2$. The following day, medium was gently removed and replaced with 4 ml of viral supernatant, supplemented as described. The plate was centrifuged a third time and returned to 32°C / 5% $CO_2$ for 5 hours.

[051] Finally, the medium was gently removed and replaced with RPMI-20 containing IL-2 (150 IU/ml), and cells were cultured from this point onwards at 37°C / 5% $CO_2$. Figure 1 illustrates multiple eNK infections performed and visualized cytometrically by EGFP fluorescence. To the best of our knowledge, there are only two other reports documenting retroviral transduction of primary NK cells in the scientific literature (Chiorean *et al.*, 2003; Unutmaz *et al.*, 1999). However, these infections - using MSCV-based retrovirus (Chiorean *et al.*, 2003) or HIV-based lentivirus (Unutmaz *et al.*, 1999) - were limited in efficacy to the $CD56^{bright}$ NK subset. In contrast, the present approach using PINCO is broadly applicable to all NK cells, i.e., $CD56^{bright}$ NK, as well as the $CD56^{dim}$ NK subset that predominates in human peripheral blood (Cooper *et al.*, 2001). Furthermore, as shown in Figure 1, the present invention transduced cDNAs up to 3.8 kB in size, with no decrease in viral titer or NK transduction.

[052] B. Transduction of IL-2/KL Expanded Primary $CD56^{bright}$ NK cells.

[053] Alternatively to transduce the $CD56^{bright}$ NK subset, eNK were seeded at $10^6$/ml in RPMI supplemented with antibiotic/antimycotic, GlutaMAX, and 10% human AB serum (ICN), supplemented with IL-2 at 150 IU/ml and c-kit ligand (KL, Amgen) at 100 ng/ml final concentration. Following 1-week incubation at 37°C / 5% $CO_2$, cells typically expanded 3-4 fold. Whereas peripheral blood NK typically comprises two major subsets, $CD56^{bright}$ (5-15% of NK) and $CD56^{dim}$ (85-95% of NK), this weeklong culture in IL-2 and KL enriches massively for the $CD56^{bright}$ subset, to the point that it represents 90% or more of the total cell number. Between $1\text{-}5x10^6$ IL-2/KL expanded NK were transduced with PINCO as described above, except that KL was included throughout the infection period at 100 ng/ml final concentration. This reproducibly resulted in profoundly high levels of infection, as illustrated in Figure 2.

[054] C. Transduction of NK-derived cell lines.

[055] The NK-92 cell line was obtained from the American Tissues Type Culture Center (ATCC, Rockville, Maryland). The NKL cell line was obtained from Jerome Ritz (Harvard University). Both lines were cultured in RPMI-20 supplemented with IL-2 (Roche, 150 IU/ml) at 37°C / 5% $CO_2$. On the day before infection, IL-2 concentration was adjusted to 900 IU/ml. The infections were performed as described in Section A. Following the final round of infection, the IL-2 concentration was returned to 150 IU/ml. This routinely results in high levels of infection, as shown in Figure 3 for NK-92 and Figure 4 for NKL. Similar results have been reported in the scientific literature (Chiorean *et al.*, 2003; Kikuchi-Maki *et al.*, 2003), using MSCV-based transfer vectors. To the best of our knowledge, however,

this is the first report of PINCO's use as a gene delivery vector for NK-derived cell lines.

[056] III. Further Analysis of Retrovirally Transduced Cells.

[057] Following retroviral infection of primary NK and NK-derived cell lines, the transduced populations were routinely isolated to >99% purity by fluorescence-assisted cell sorting (FACS) for $GFP^+$ cells. When virally transduced NK-92 and NKL were subject to long-term culture (>3 months), no significant decrease in GFP fluorescence was observed. As an alternative to GFP, expression of proteins of interest was monitored directly by immunoblotting. Indeed, Western blot analysis of FACS purified, virally transduced NK cell lines reveals significant overexpression of these proteins, as shown in Figure 6.

[058] As shown in Figure 7, retrovirally transduced NK cells are capable of robust IFN-γ production, as shown both by ELISA and by intracellular flow cytometric analysis. Note in particular that retrovirus infection itself does not have any deleterious effect on this parameter of NK cell function, as revealed by equivalent IFN-γ production by uninfected and infected populations in Figure 6A. Finally, virally transduced cells also exhibit strong cytolytic activity toward traditional NK target cells (e.g., K-562) in $^{51}Cr$-release assays (Figure 8). In sum, transduction of NK cells and NK-derived cell lines with PINCO retrovirus preserves cellular functions, including cytolysis and cytokine elaboration.

[059] This technical advance paves the way for mechanistic studies of the roles of individual genes in NK cell function. In addition, with the advent of NK-cell transplantation in cancer therapy for patients with acute myeloid leukemia, the

genetic manipulation of NK cell populations prior to administration may conceivably provide therapeutic benefit for the patient - by enhancing NK-cell survival, cytolytic function, cytokine production, and/or tumor-specific killing.

[060] IV. Use of CD8 as a Sorting Facilitator

Rather than use GFP as a marker for NK transduction, we have substituted a modified mouse CD8 mRNA, which encodes a cytoplasmically truncated protein that is targeted to the cell membrane but which lacks signaling properties, rendering this molecule inert. Since the extracellular domain of the CD8 is preserved, transduced NK-92 were detected by flow cytometry using well-established monoclonal antibodies to this molecule, which are conjugated to fluorescence molecules. The use of CD8 and GFP in separate viruses permits infection of NK cells with more than one gene. Furthermore, the use of CD8 allows the possibility to isolate transduced cells using magnetic beads pre-conjugated to anti-CD8, eliminating the need to rely on fluorescence-assisted cell sorting (FACS) for purification of the transduced population.

V. RNAi EXAMPLE

RNA interference (RNAi) is a method for silencing expression of specific gene(s) of interest based on the transduction of small, double-stranded RNA sequences identical to the mRNA of the particular gene(s). We modified PINCO to express a CD8 along with an RNAi cassette from the pSUPER plasmid (OligoEngine, Seattle, WA), which was previously engineered to express a short hairpin RNA (shRNA) that targets a specific gene, T-BET. Transduction of NK cells results in the expression of CD8 (as a marker of infection) and the shRNA from

separate promoters. The shRNA, which contains complementary regions to permit folding on itself to form a hairpin, is subsequently processed by cellular enzymes to form a double-stranded RNA that targets and silences the gene of interest.

VI. Citations

[061] Chiorean EG, Dylla SJ, Olsen K, Lenvik T, Soignier Y, Miller JS. BCR/ABL alters the function of NK cells and the acquisition of killer immunoglobulin-like receptors (KIRs). Blood. 2003 May 1;101(9):3527-33.

[062] Cooper MA, Fehniger TA, Turner SC, Chen KS, Ghaheri BA, Ghayur T, Carson WE, Caligiuri MA. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood. 2001 May 15;97(10):3146-51.

[063] Fehniger TA, Cooper MA, Caligiuri MA. Interleukin-2 and interleukin-15: immunotherapy for cancer. Cytokine Growth Factor Rev. 2002 Apr;13(2):169-83.

[064] Grignani F, Kinsella T, Mencarelli A, Valtieri M, Riganelli D, Grignani F, Lanfrancone L, Peschle C, Nolan GP, Pelicci PG. High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. Cancer Res. 1998 Jan 1;58(1):14-9.

[065] Introna M, Barbui AM, Golay J, Bambacioni F, Schiro R, Bernasconi S, Breviario F, Erba E, Borleri G, Barbui T, Biondi A, Rambaldi A. Rapid retroviral infection of human haemopoietic cells of different lineages: efficient transfer in fresh T cells. Br J Haematol. 1998 Nov;103(2):449-61.

[066] Kikuchi-Maki A, Yusa S, Catina TL, Campbell KS. KIR2DL4 is an IL-2-regulated NK cell receptor that exhibits limited expression in humans but triggers strong IFN-gamma production. J Immunol. 2003 Oct 1;171(7):3415-25.

[067] M. Sattler, S. Verma, C. H. Byrne, G. Shrikhande, T. Winkler, P. A. Algate, L. R. Rohrschneider, and J. D. Griffin. BCR/ABL Directly Inhibits Expression of SHIP, an SH2-Containing Polyinositol-5-Phosphatase Involved in the Regulation of Hematopoiesis. Mol. Cell. Biol., November 1, 1999; 19(11): 7473 - 7480.

[068] Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F, Martelli MF, Velardi A. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002 Mar 15;295(5562):2097-100.

[069] Trompeter HI, Weinhold S, Thiel C, Wernet P, Uhrberg M. Rapid and highly efficient gene transfer into natural killer cells by nucleofection. J Immunol Methods. 2003 Mar 1;274(1-2):245-56.

[070] Unutmaz D, KewalRamani VN, Marmon S, Littman DR. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. J Exp Med. 1999 Jun 7;189(11):1735-46.

[071] Except where otherwise indicated, all numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should

be construed in light of the number of significant digits and ordinary rounding approaches.

[072] The specification is most thoroughly understood in light of the teachings of the references cited within the specification, all of which are hereby incorporated by reference in their entirety. The embodiments within the specification provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention. The skilled artisan recognizes that many other embodiments are encompassed by the claimed invention and that it is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

WHAT IS CLAIMED IS:

1. A method for stably transducing mammalian natural killer cells comprising:

transfecting a packaging cell line with a retroviral expression vector;

culturing the transfected packaging cell line in a cell culture medium; and

culturing the mammalian natural killer cells with the cell culture medium.

2. The method according to claim 1, wherein the packaging cell line is chosen from a Phoenix cell line.

3. The method according to claim 2, wherein the packaging cell line is Phoenix-Ampho.

4. The method according to claim 1, wherein the retroviral expression vector is PINCO.

5. The method according to claim 1, further comprising separating the transfected packaging cell line from the cell culture medium in which the cell line is cultured prior to culturing the mammalian natural killer cells with the cell culture medium.

6. A non-naturally occurring mammalian natural killer cell, stably transduced, which expresses at least one of green fluorescent protein and CD8.

7. A progeny cell line of the non-naturally occurring mammalian natural killer cell according to claim 6, wherein the cell line is polyclonal.

8. A non-naturally occurring mammalian natural killer cell line, which stably expresses its genome through at least two population doublings.

9. The non-naturally occurring mammalian natural killer cell line according to claim 8, which stably expresses its genome through at least four population doublings.

10. The non-naturally occurring mammalian natural killer cell line according to claim 9, which stably expresses its genome through at least eight population doublings.

## ABSTRACT OF THE DISCLOSURE

Methods for stably transducing mammalian natural killer cells comprising: transfecting a packaging cell line with a retroviral expression vector; culturing the transfected packaging cell line in a cell culture medium; and culturing the mammalian natural killer cells with the cell culture medium. Natural killer cells transduced according to the disclosed methods are also provided.

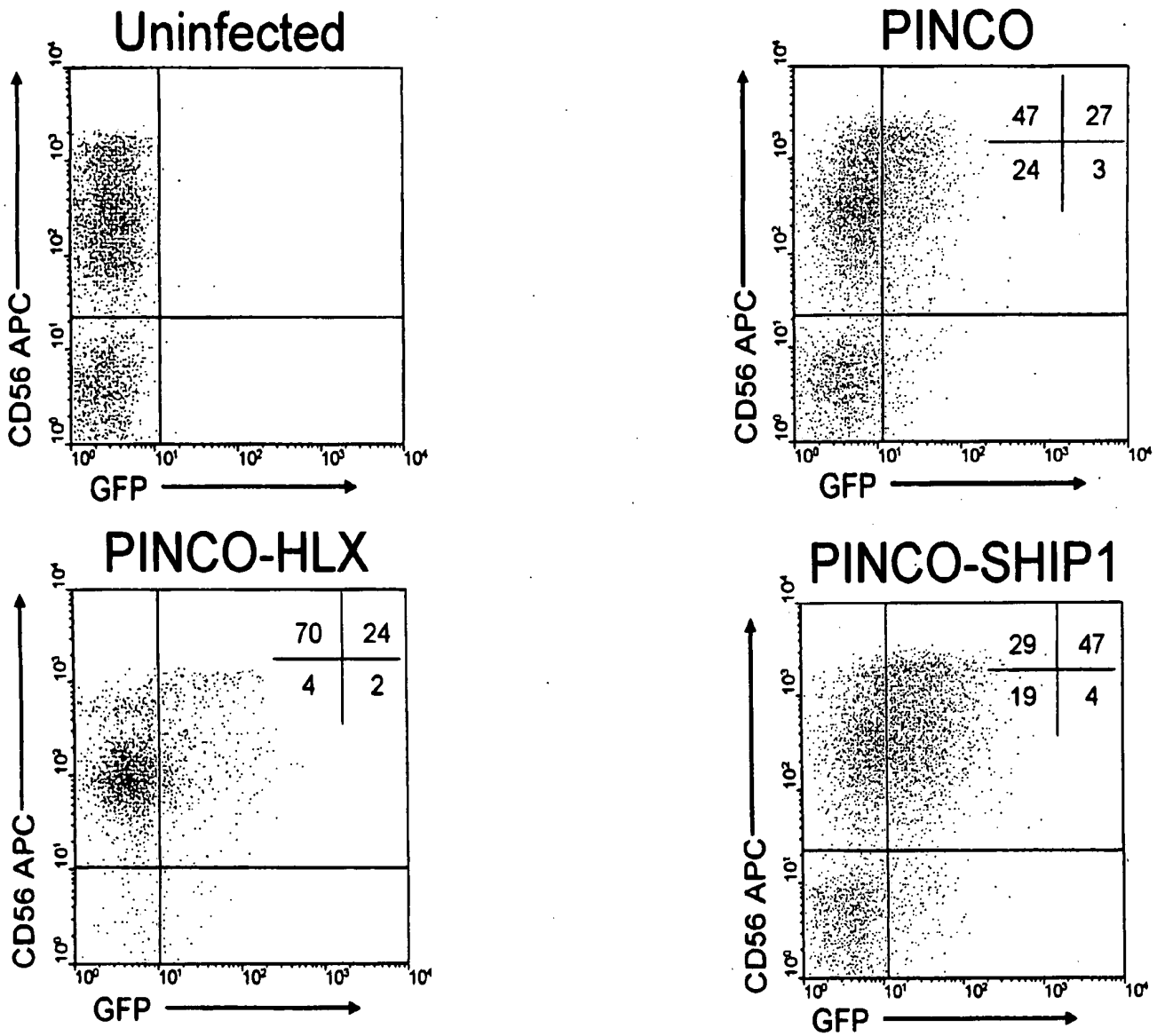

Uninfected
PINCO
47
27
24
3
PINCO-HLX
70
24
4
2
PINCO-SHIP1
29
47
19
4
CD56 APC
GFP

**Figure 1**

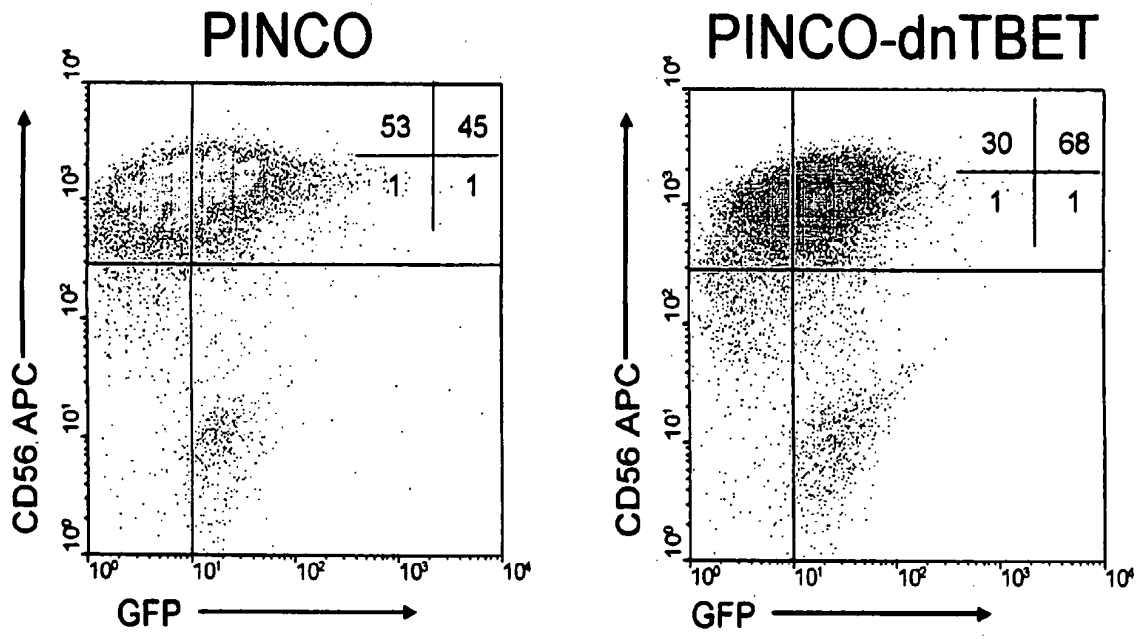

PINCO
PINCO-dnTBET
CD56 APC
GFP
53
45
1
1
30
68
1
1
10⁰
10¹
10²
10³
10⁴

**Figure 2**

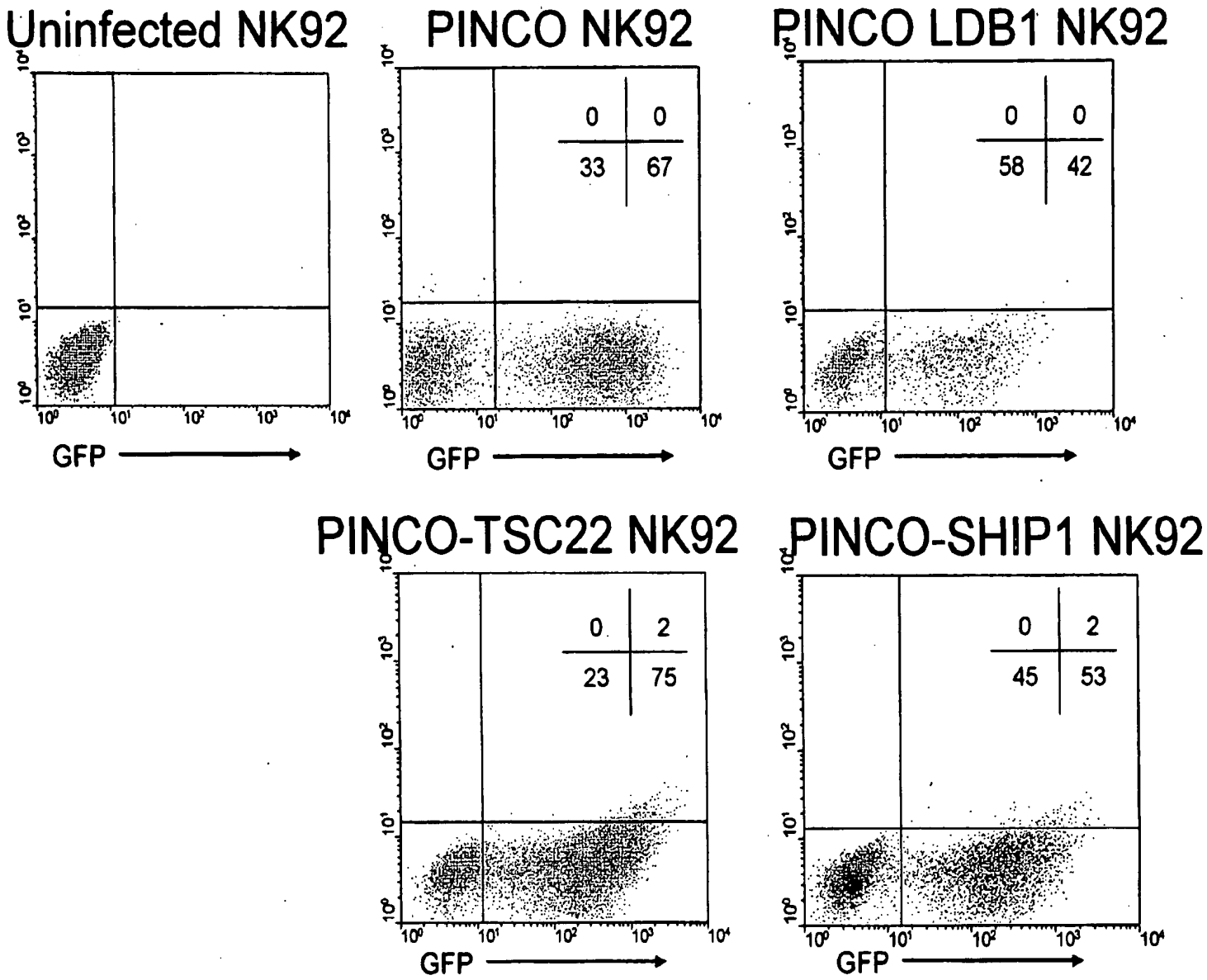
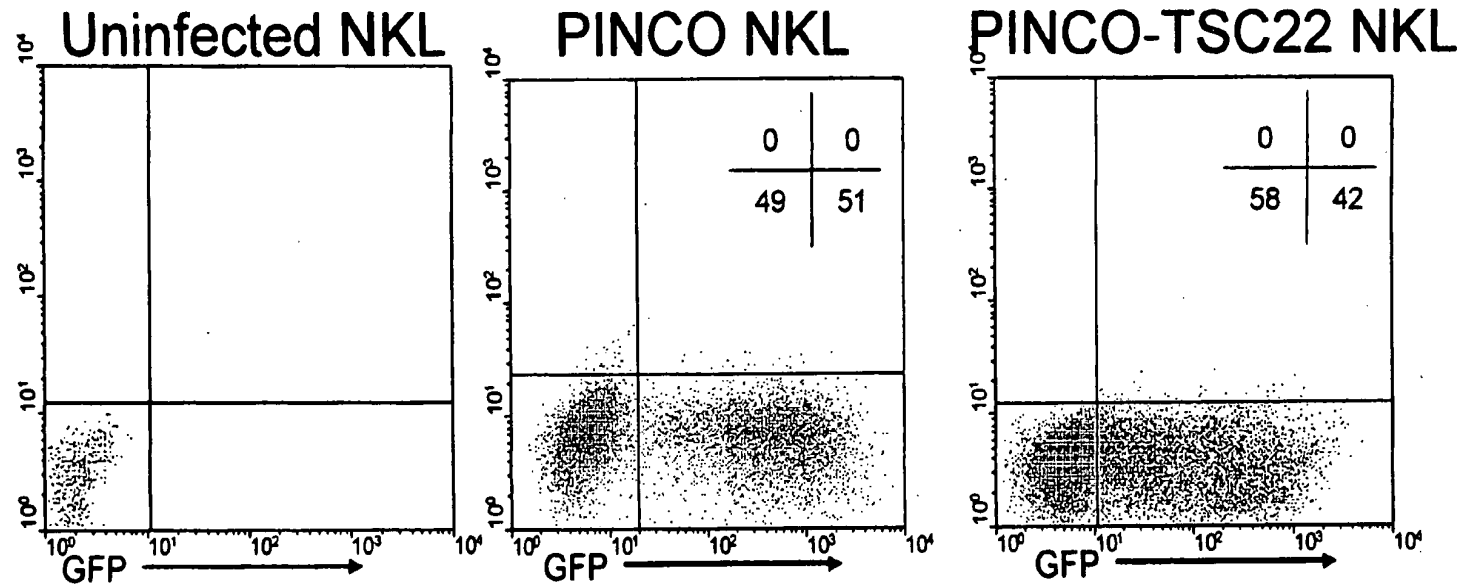

Uninfected NK92
PINCO NK92
PINCO LDB1 NK92
PINCO-TSC22 NK92
PINCO-SHIP1 NK92
0
0
33
67
0
0
58
42
0
2
23
75
0
2
45
53
GFP

**Figure 3**


Uninfected NKL
PINCO NKL
PINCO-TSC22 NKL
0
0
49
51
0
0
58
42
GFP
GFP
GFP

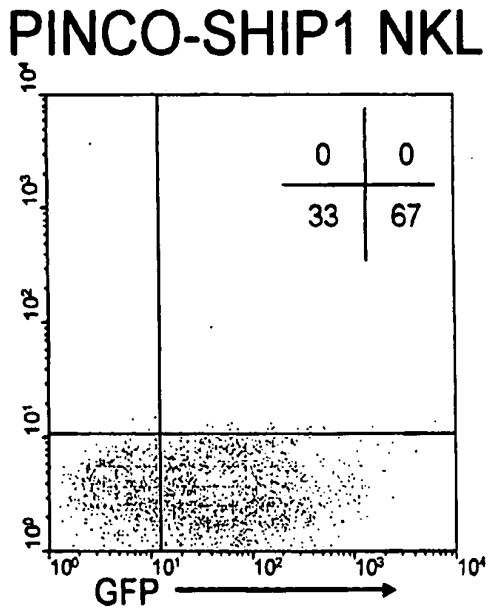
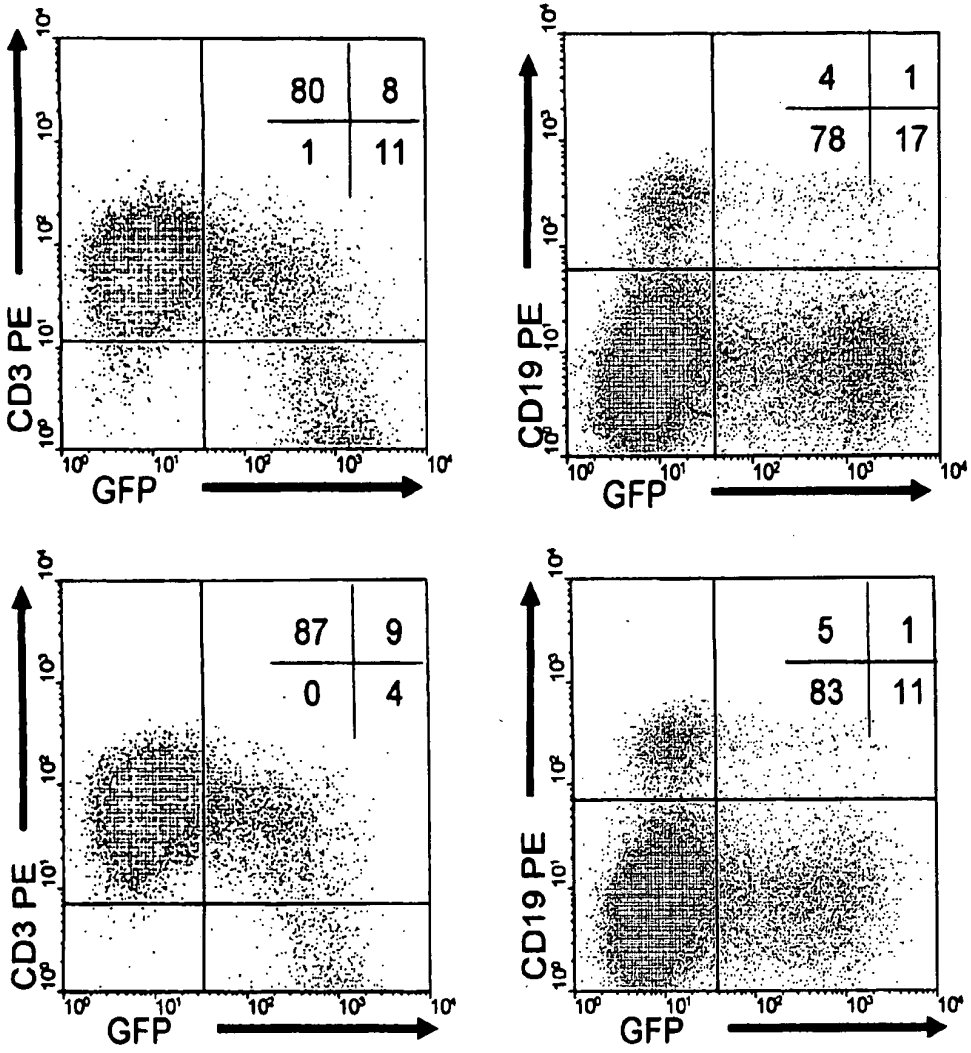

PINCO-SHIP1 NKL
0
0
33
67
GFP

**Figure 4**




PINCO PBMC

PINCO-TSC22 PBMC

**Figure 5**

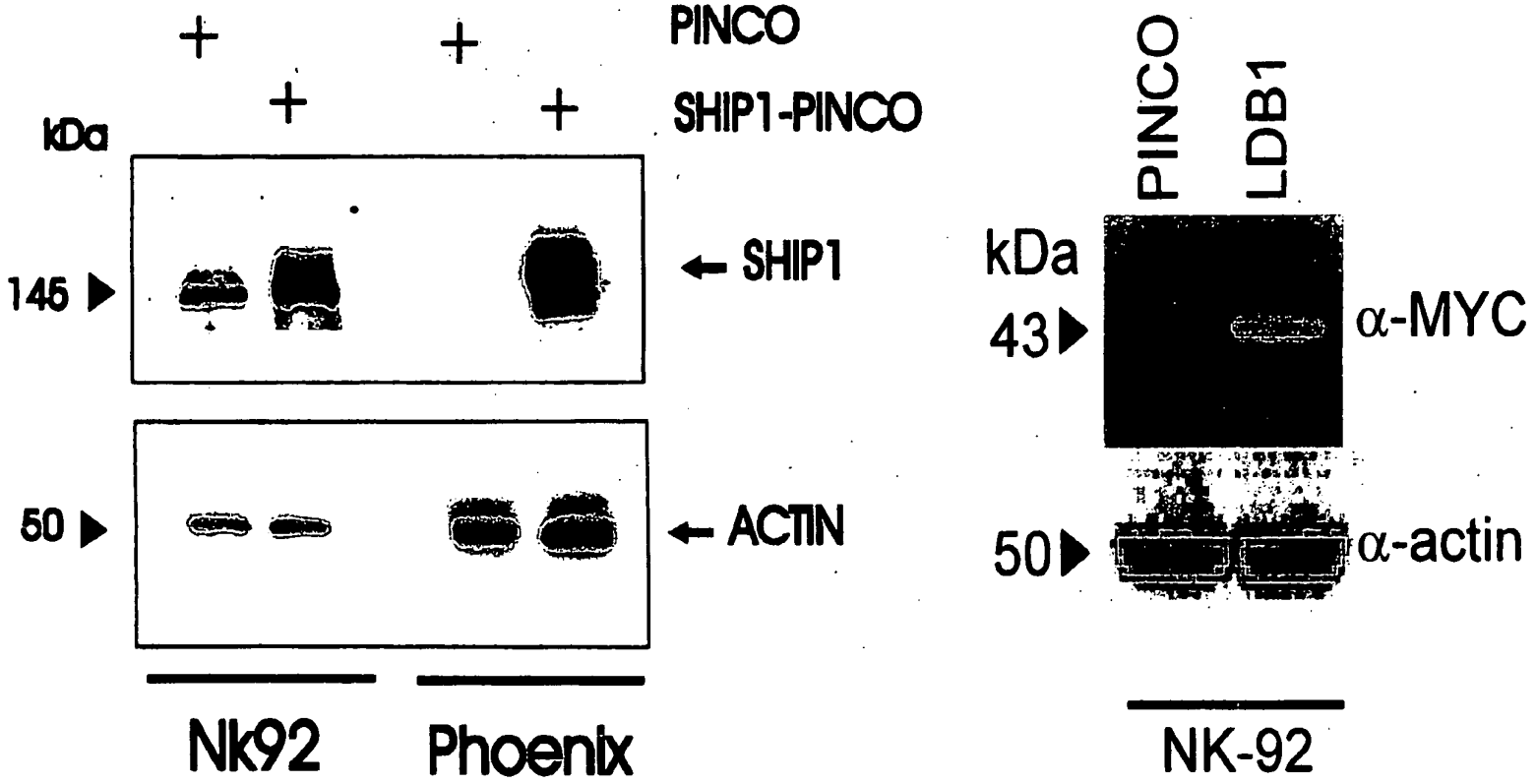

+
+
PINCO
+
+
SHIP1-PINCO
kDa
145
SHIP1
50
ACTIN
Nk92
Phoenix
PINCO
LDB1
kDa
43
α-MYC
50
α-actin
NK-92

**Figure 6**

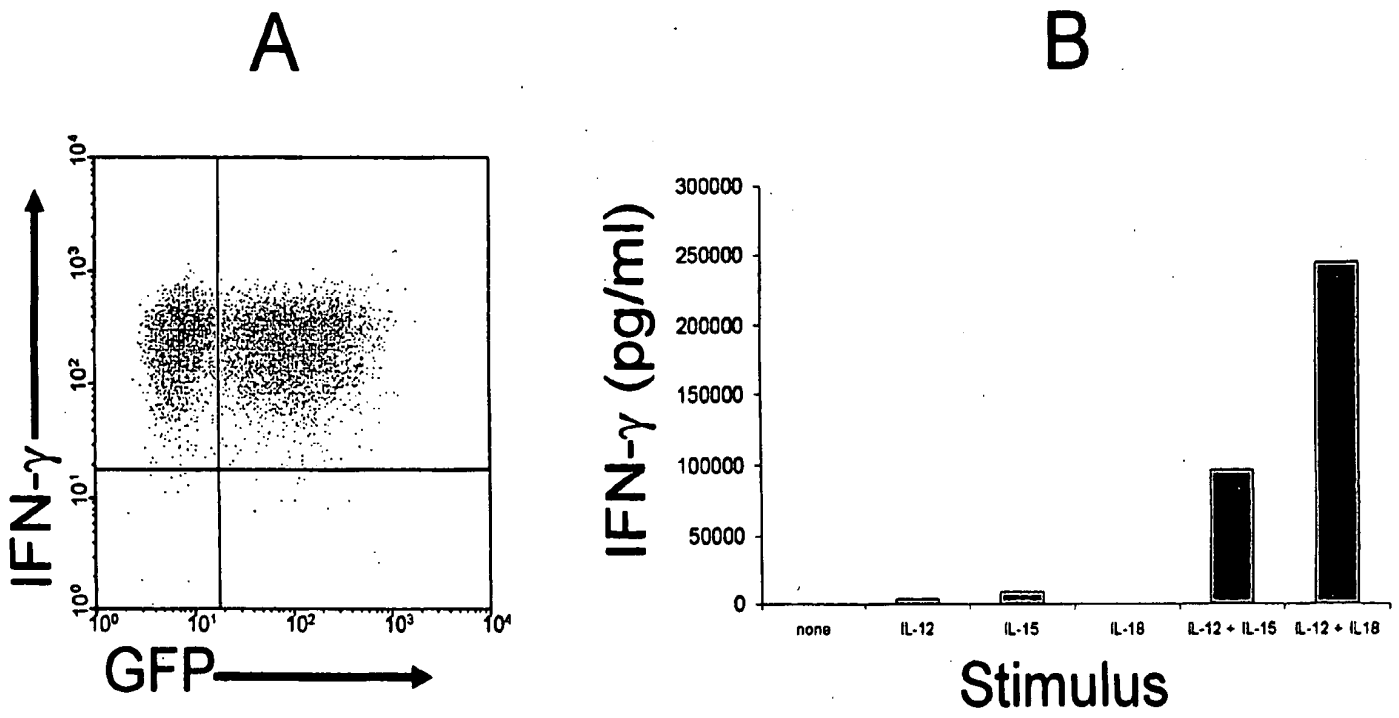

A
B
IFN-γ
GFP
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
IFN-γ (pg/ml)
300000
250000
200000
150000
100000
50000
0
none
IL-12
IL-15
IL-18
IL-12 + IL-15
IL-12 + IL18
Stimulus

**Figure 7**

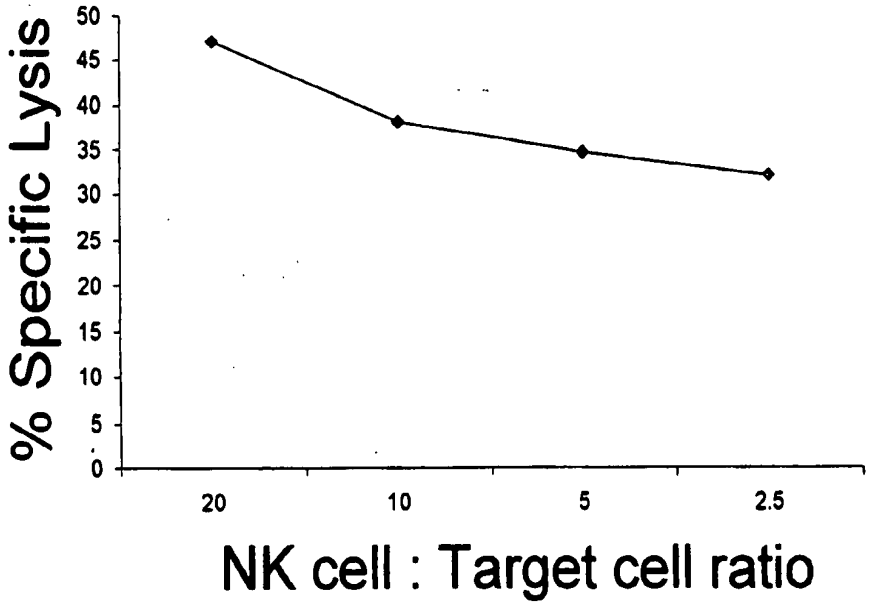

% Specific Lysis
50
45
40
35
30
25
20
15
10
5
0
20
10
5
2.5
NK cell : Target cell ratio

**Figure 8**

# PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

# PCT

NOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

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| | |
|---|---|
| Date of mailing *(day/month/year)*<br>25 May 2005 (25.05.2005) | |
| Applicant's or agent's file reference<br>22727/04309 | **IMPORTANT NOTIFICATION** |
| International application No.<br>PCT/US05/009238 | International filing date *(day/month/year)*<br>21 March 2005 (21.03.2005) |
| International publication date *(day/month/year)* | Priority date *(day/month/year)*<br>22 March 2004 (22.03.2004) |
| Applicant<br>THE OHIO STATE UNIVERSITY RESEARCH FOUNDATION et al | |

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2. *(If applicable)* The **letters "NR"** appearing in the right-hand column denote **a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau** under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

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| Priority date | Priority application No. | Country or regional Office or PCT receiving Office | Date of receipt of priority document |
|---|---|---|---|
| 22 March 2004 (22.03.2004) | 60/555,177 | US | 09 May 2005 (09.05.2005) |

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